Effects of Ketoconazole on Progesterone and cAMP Production in MA-10 Mouse Leydig Tumor Cells

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The effects of ketoconazole (KCZ) on secretion of progesterone and cAMP in Leydig cells were investigated in vitro. MA-10 mouse Leydig tumor cells were used to conduct the experiments. KCZ significantly inhibited the progesterone production from MA-10 cells in a dose dependent fashion between 2 and 20 μM among 1, 2 and 3 h of incubation. There was a statistically significant difference in luteinizing hormone (LH) stimulated progesterone production inhibited by 2 and 20 μM KCZ treatment compared to the control. The effect of KCZ on progesterone biosynthesis in MA-10 cells was mediated by cAMP, since KCZ suppressed basal and LH stimulated cAMP production and content within the same dose range. The stimulatory effects of forskolin and sodium fluoride on the adenylate cyclase system were also inhibited by KCZ. Moreover, dibutyryl cAMP blocked the inhibitory effect on steroidogenesis of KCZ in MA-10 cells. These data indicated that KCZ induced the inhibition of a catalytic component of adenylate cyclase holoenzyme in MA-10 mouse Leydig tumor cells.

Key words ketoconazole; progesterone; cAMP; Leydig cell

The imidazole derivative, ketoconazole (KCZ), an orally active antifungal and anticycotic drug, inhibits the synthesis of ergosterol in fungal membrane, and of cholesterol in mammalian cells. It inhibits adrenal steroidogenesis and blunts testosterone production. KCZ is a competitive inhibitor of cytochrome P450-dependent enzymes, mainly inhibiting the enzyme activity of the cholesterol side-chain-cleavage enzyme and 3β-hydroxysteroid dehydrogenase. Because KCZ acts as a steroid hormone inhibitor with differential selectivity of cytochrome P-450 dependent enzymes, it is a therapeutic tool in the management of conditions in which it is beneficial to suppress gonadal or adrenal hormone production, such as advanced hormone-refractory prostate cancer and Cushings syndrome.

Leydig cells secrete testosterone, an essential steroid hormone in male reproductive functions. A MA-10 mouse Leydig tumor cell line was utilized for this study. MA-10 cells, responding to trophic hormones (LH and hCG) and cAMP analogs, produce progesterone as the main steroid hormone instead of testosterone. Moreover, the use of this cloned cell line may avoid the presence of paracrine factors from other testicular cells of primary cultures. The purpose of the present study was to further characterize the effects of KCZ on secretion of progesterone and cAMP production in MA-10 cells and to determine the mechanism in vitro.

MATERIALS AND METHODS

Materials Ketoconazole, luteinizing hormone (LH), 3-isobutyl-1-methylxanthine (IBMX), forskolin, sodium fluoride, dibutyryl-adenosine-3':5'-cyclic monophosphate (db-cAMP) and all other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Weymouth medium, horse serum and cell culture related materials were purchased from Gibco-BRL (Grand Island, NY, U.S.A.). Materials related to progesterone enhanced chemiluminescent detection were purchased from Ortho-Clinical Diagnostics Inc., a Johnson & Johnson company (Rochester, NY, U.S.A.). Materials related to the cAMP enzyme immunoassay (EIA) system and BIOTRAK cellular communication assays were purchased from Amersham Pharmacia Biotech (Buckinghamshire, England, U.K.).

Cell Culture and Samples Detection The MA-10 cell line was generously provided by Dr. Ing-Cherng Guo (National Taiwan University, Taipei, Taiwan R.O.C.) and Dr. Bon-Chu Chung (Academia Sinica, Taipei, Taiwan R.O.C.) and has been maintained since an earlier study. 5×10⁴ cells/ml medium or 5×10⁵ cells/100 μl medium were plated in 24-well plates or 96-well plates, respectively. Cells were grown for 24 h in Weymouth medium containing 15% horse serum. Then, the medium was aspirated and the cells were washed with phosphate buffer saline (PBS). Chemicals and/or KCZ were continuously added to the serum-free Weymouth medium at various concentrations and times. Cells were maintained in a humidified incubator containing 5% CO₂ at 37°C. After incubation, media were collected and progesterone concentrations and cAMP levels in each treatment were determined by chemiluminescent detection and cAMP EIA, respectively. Progesterone samples were measured by the detector, VITROS Eci, Ortho-Clinical Diagnostics Inc., a Johnson & Johnson company (Rochester, NY, U.S.A.).

Statistics All experiments, with duplicates of each treatment, were repeated at least three times. Data of all experiments were analyzed by analysis of variance (ANOVA), and then the least significant difference (LSD) was used to determine the difference between treatments.

RESULTS

Dose and Time Course Effects of KCZ on Progesterone Production in MA-10 Cells The effect of different incubation times (1, 2, 3 h) with different concentrations of KCZ (2, 20 μM) on progesterone production in MA-10 cells is shown in Fig. 1. The progesterone levels after 20 μM KCZ treatment were lower than those after 2 μM KCZ treatment. When incubated with 20 μM KCZ, the production of progesterone decreased at least 6-fold compared with the basal level. Meanwhile, significant difference in progesterone production was suppressed by KCZ compared with the basal level from 1 to
3 h incubation, especially those values since 2 h after KCZ treatment.

**Effect of KCZ on Progesterone Production in MA-10 Cells after LH Stimulation** The effect of different LH dosages (5, 25 μg/ml) on progesterone secretion in MA-10 cells without and with KCZ incubation (2, 20 μM) is shown in Fig. 2. Pretreatment (2 h) with 2 and 20 μM KCZ led to an inhibition of basal progesterone secretion and also of progesterone secretion after stimulation with different LH dosages (5, 25 μg/ml; Fig. 2). Progesterone increased 80-fold (from 0.76±0.05 to 61.87±2.93 nmol/l) 2 h after 25 μg LH/ml without KCZ, whereas pretreatment with 20 μM KCZ decreased basal and LH-stimulated progesterone production (0.11±0.02, 9.15±2.90 nmol/l). This pattern was noted with all LH and KCZ dosages.

**cAMP Production and Progesterone Secretion of Rela-

tive Tests** The cAMP levels corresponded to the progesterone production in MA-10 cells. Basal cAMP level increased 1.2-fold after 5 μg LH/ml stimulation (from 322±13 fmol/5×10^4 cells to 393±14 fmol/5×10^4 cells) and after inhibition of phosphodiesterase with 0.5 mM IBMX. Basal cAMP level increased 1.01±0.02 μM after 5 μg LH/ml stimulation (from 55.60±3.39 μM to 65.60±3.43 μM). This pattern was noted with all LH and KCZ dosages.

**Table 1. Effect of KCZ and db-cAMP on Progesterone Production in MA-10 Cells**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Progesterone (nmol/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal</td>
<td>0.76±0.05</td>
</tr>
<tr>
<td>KCZ (20 μM)</td>
<td>0.11±0.02*</td>
</tr>
<tr>
<td>LH (5 μg/ml)</td>
<td>55.60±3.39*</td>
</tr>
<tr>
<td>LH+KCZ</td>
<td>2.45±0.55</td>
</tr>
<tr>
<td>KCZ+db-cAMP (10 μM)</td>
<td>3.43±0.41</td>
</tr>
<tr>
<td>LH+KCZ+db-cAMP</td>
<td>12.80±0.93</td>
</tr>
</tbody>
</table>

MA-10 cells were incubated 24 h. Different compounds were added to selected wells and aliquots of the medium were removed from well at 2 h. Progesterone in the medium was measured by enhanced chemiluminescent detection. Each piece of data in the table represents mean ± S.E. of progesterone production of repeated experiments with triplicates in each treatment. Asterisk (*) indicates statistically significant difference (p<0.05) among treatments.

**Fig. 1.** The Inhibitory Effect of KCZ Incubation (Basal, 2, 20 μM) on Progesterone Production in MA-10 Cells for Different Periods (1—3 h).

MA-10 cells were incubated 24 h. Progesterone in the medium was measured by enhanced chemiluminescent detection. Each data point in the figure represents mean ± S.E. of progesterone production of repeated experiments with triplicates in each treatment. Different Arabic numbers beside the lines indicates statistically significant differences (p<0.05) among treatments.

**Fig. 2.** The Inhibitory Effect of KCZ Incubation (Basal, 2, 20 μM) on Progesterone Production in MA-10 Cells with Basal and LH Stimulation (Basal, 5, 25 μg/ml for 2 h).

KCZ was preincubated for 2 h. MA-10 cells were incubated 24 h. Progesterone in the medium was measured by enhanced chemiluminescent detection. Each data point in the figure represents mean ± S.E. of progesterone production of repeated experiments with triplicates in each treatment. Asterisk (*) indicates statistically significant difference (p<0.05) as compared with control (without KCZ pretreatment) levels.

**Fig. 3.** The Inhibitory Effect of KCZ Incubation (Basal, 20 μM) on cAMP Production and Corresponding Progesterone Secretion in MA-10 Cells with IBMX (0.5 mM) as Phosphodiesterase Inhibitor, LH (5 μg/ml) or Both for 2 h.

KCZ was preincubated for 2 h. MA-10 cells were incubated 24 h. cAMP in the medium was measured by EIA. Progesterone in the medium was measured by enhanced chemiluminescent detection. Each data point in the figure represents mean ± S.E. of cAMP and progesterone production of repeated experiments with triplicates in each treatment. Asterisk (*) indicates statistically significant difference (p<0.05) as compared with control (without KCZ pretreatment) levels. Arabic numbers above the bars indicates corresponding progesterone secretion. 1, 0.62±0.05; 2, 61.40±7.86; 3, 4.31±0.70; 4, 3.59±0.80; 5, 0.08±0.01; 6, 0.36±0.10; 7, 4.70±0.69; 8, 3.29±0.45 nmol/l.
pendent inhibition of progesterone synthesis by KCZ was MA-10 mouse Leydig tumor cells (Fig. 1). Similar dose-de-

progression in a dose dependent fashion from 1 to 3 h in

(Table 2).

nificantly suppressed by 20

ride (1 mM) incubation which stimulated the G\textsubscript{S} protein sig-

nificant progesterone and cAMP production, which was also

significant inhibition of phosphodiesterase with

was measured by enhanced chemiluminescent detection. cAMP in the medium was

and aliquots of the medium were removed from well at 2 h. Progesterone in the medium

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Table 2. Effect of KCZ on Forskolin and Sodium Fluoride Stimulated Progesterone and cAMP Production in MA-10 Cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Progesterone (nmol/l)</th>
<th>cAMP (fmol/5\times10^5 cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal</td>
<td>0.6±0.04</td>
<td>202±10</td>
</tr>
<tr>
<td>Forskolin (5 \muM)</td>
<td>2.9±0.24\textsuperscript{*}</td>
<td>399±13\textsuperscript{*}</td>
</tr>
<tr>
<td>Forskolin + KCZ (20 \muM)</td>
<td>0.42±0.11\textsuperscript{**}</td>
<td>270±9\textsuperscript{**}</td>
</tr>
<tr>
<td>Basal</td>
<td>0.70±0.08</td>
<td>195±4</td>
</tr>
<tr>
<td>Sodium fluoride (1ms)</td>
<td>2.13±0.21\textsuperscript{*}</td>
<td>331±20\textsuperscript{*}</td>
</tr>
<tr>
<td>Sodium fluoride + KCZ (20 \muM)</td>
<td>0.24±0.07\textsuperscript{*}</td>
<td>229±13\textsuperscript{*}</td>
</tr>
</tbody>
</table>

MA-10 cells were incubated 24h. Different compounds were added to selected wells and aliquots of the medium were removed from well at 2h. Progesterone in the medium was measured by enhanced chemiluminescent detection. cAMP in the medium was measured by EIA. Each data in the table represents mean±S.E. of progesterone and cAMP production of repeated experiments with triplicates in each treatment. Asterisk (\textsuperscript{*} \textsuperscript{**}) indicates statistically significant difference (p<0.05) as compared with control (without KCZ pretreatment) levels. Different letters beside the values indicates statistically significant difference (p<0.05) among treatments.

20 \muM KCZ (Table 1). Stimulation of the catalytic subunit of the adenylate cyclase system with 5 \muM forskolin led to significant progesterone and cAMP production, which was also significantly inhibited by 20 \muM KCZ (Table 2). Sodium fluoride (1 mM) incubation which stimulated the G\textsubscript{S} protein significantly increased progesterone production which was significantly suppressed by 20 \muM KCZ. Meanwhile, cAMP releases after 1 mM sodium fluoride treatment were significantly increased and significantly suppressed by 20 \muM KCZ (Table 2).

DISCUSSION

Our data demonstrated that KCZ inhibits secretion of progesterone in a dose dependent fashion from 1 to 3 h in MA-10 mouse Leydig tumor cells (Fig. 1). Similar dose-de-
pended inhibition of progesterone synthesis by KCZ was shown in a previous study,\textsuperscript{22} KCZ inhibited the hCG- and db-cAMP-stimulated progesterone production from MA-10 Leydig cells by directly inhibiting the activity of cholesterol side-chain cleavage enzyme.\textsuperscript{22} Inhibition of \textsubscript{C\textsubscript{17—20}} lyase by KCZ has been demonstrated by an increase in the ratio of precursor (17\textalpha-hydroxyprogesterone or 17\textalpha,20\textalpha-dihydroxy-progesterone or progesterone or all) to product (androstenedione or testosterone) both \textit{in vivo}\textsuperscript{13,16} and \textit{in vitro}.\textsuperscript{23—25} Moreover, cholesterol side-chain-cleavage blockade by KCZ has been demonstrated in both testicular and adrenal tissue preparations.\textsuperscript{26,27} Therefore, the significantly inhibited progesterone production in MA-10 cells by KCZ corresponds to the results of \textit{in vitro} studies in rat testicular microsomes with KCZ. It has shown that cholesterol side-chain-cleavage has a higher sensitivity to KCZ than does \textsubscript{C\textsubscript{17—20}} lyase.\textsuperscript{27} MA-10 cells released progesterone into the medium in response to different doses of LH (Fig. 2); this release was coupled with an increase of cAMP levels (Fig. 3).\textsuperscript{28} That cAMP is the second messenger to LH stimulation of progesterone in MA-10 Leydig tumor cells was demonstrated by the observation that added db-cAMP also led to a rise in progesterone production. Moreover, inhibition of phosphodiesterase with IBMX\textsuperscript{29} led to an increase in cAMP production and progesterone secretion.

The stimulation of the catalytic component of the adenylate cyclase holoenzyme with forskolin\textsuperscript{30} also induced a significant increase in cAMP and progesterone production in MA-10 cells. A stimulatory agent of the G\textsubscript{S} protein of adenylate cyclase system, like sodium fluoride,\textsuperscript{30} also led to an increase in progesterone and cAMP production in MA-10 cells. KCZ suppressed basal cAMP and progesterone production from these cells (Figs. 1—3). Basal phosphodiesterase activity was inhibited by IBMX\textsuperscript{29} indicating that adenylate cyclase is required to maintain progesterone biosynthesis which is inhibited by KCZ (Fig. 3). Therefore, KCZ inhibited progesterone secretion and cAMP production after inhibiting of phosphodiesterase by IBMX and stimulating by different doses of LH (Figs. 2, 3). MA-10 cells have LH/hCG receptors and respond to LH/hCG and cAMP via increased production of progesterone.\textsuperscript{21,22} KCZ decreased cAMP production by blocking the pathway of catalytic component of the adenylate cyclase in the present study, and was also an inhibitor of the cholesterol side-chain-cleavage enzyme.\textsuperscript{1—9,11} Therefore, the significant inhibitory effect of KCZ incubation on LH-stimulated progesterone production in MA-10 cells was not noted in combined IBMX treatment (Fig. 3). However, the reason a lower extent of increased progesterone values did not match the higher cAMP levels after IBMX treatment needs further investigation. In contrast, the hCG-stimu-
lated cAMP production was not inhibited by KCZ.\textsuperscript{22} Chaudhary and Stocco suggested that the locus of hCG-stimulated progesterone synthesis was not related to cAMP production in MA-10 cells.\textsuperscript{22} This meant that the inhibitory effects of KCZ between progesterone and cAMP production after LH stimulation (Figs. 2, 3) interacted in MA-10 cells. Addition of exogenous db-cAMP to MA-10 cells pretreated with KCZ neutralized the inhibitory effect of KCZ on progesterone production in the basal state and after LH stimulation (Table 1). Stimulation of the G\textsubscript{S} protein of the adenylate cyclase system by sodium fluoride was suppressed by KCZ in the same fash-

ion as the forskolin stimulated adenylate cyclase activity (Table 2).

In summary, our data suggested that KCZ inhibits cAMP generation and progesterone biosynthesis at the direction of the catalytic component of the adenylate cyclase holoenzyme in MA-10 mouse Leydig tumor cells.

REFERENCES

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